RELATIONSHIP OF p21 $^{WAF1/CIP1/SDI1}$ TO CELL PROLIFERATION IN PRIMARY CULTURES OF ADRENOCORTICAL CELLS

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ABSTRACT

p21$^{WAF1/CIP1/SDI1}$ was originally described as a protein expressed at high levels in senescent human fibroblasts. We have studied the expression of p21 in adrenocortical cells. p21 is not expressed under most circumstances in the intact adrenal gland *in vivo*, except when the gland is damaged. When human and bovine adrenocortical cells are isolated and placed in both short-term and long-term culture, p21 levels are much higher. These levels did not show a large increase when the cells senesce after long-term proliferation. Thus, these observations raise the question of whether the elevated p21 in primary cultures of adrenocortical cells is caused by damage or whether p21 is elevated because the cells are dividing rather than quiescent, because it has been reported that p21 levels peak in G1 and G2 in dividing cells. In the present experiments on bovine and human adrenocortical cells in primary culture, labeling techniques that correlated nuclear p21 with measures of cell proliferation (bromodeoxyuridine incorporation and nuclear Ki-67 antigen) supported the hypothesis that p21 is associated with cell division and not with damage. This is consistent with recent data showing that, when adrenocortical cells are transplanted into immunodeficient mice, p21 is associated with healthy dividing cells in the transplant. p21 is not a unique marker for senescence, and more studies are required both to clarify its role in cell biology and to determine molecular features which characterize the senescent state of cells both in vitro and in vivo.

INTRODUCTION

p21$^{WAF1/CIP1/SDI1}$ is a multifunctional protein that inhibits cyclin-dependent kinases (CDKs) and blocks cycling of cells (1 - 4). One major role is to act as a mediator of the action of p53 in response to DNA damage, being responsible for p53-dependent growth arrest under those circumstances where p53 causes arrest rather than apoptosis (5, 6). However, in undamaged cells growing in culture there are peaks of p21 in both the G1 and G2

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phases of the cell cycle (7, 8). On the other hand, quiescent cells that are completely out of the cell cycle may have very low p21; most cells *in vivo* are quiescent and also do not express p21 (9).

Our interest in p21 began when it was discovered by Dr. James R. Smith as a protein expressed at high levels in senescent human fibroblasts (1, 4). In the time since its discovery, several hundred publications have documented the role of the p21 in the biology of mammalian cells. Most of those experiments have been in cells in culture.

In this laboratory, we have developed methods for studying growth, gene expression, and senescence of a differentiated cell type, the adrenocortical cell. Because we are able to isolate a pure population of cells freshly from the adrenal cortex, and plate them into primary culture, we are able to directly compare the biology of the cells *in vivo* with their biology in both short-term and long-term culture (10). More recently, we have extended this system by showing that the cells may be transplanted into immunodeficient mice, where they form a functional, vascularized tissue structure (11, 12). The combination of these *in vitro* and *in vivo* models provides a unique opportunity to study how proliferation, differentiation and senescence are regulated when cells are in these very different environments.

Because of the role of p21 in the senescence of human fibroblasts, and because of its importance as a cell cycle regulator, we studied the expression of p21 in adrenocortical cells under different *in vitro* and *in vivo* conditions. We found very little expression of p21 in the intact human, rat, and bovine adrenal cortex, using immunohistochemistry, Northern blotting, and Western blotting (13 - 15). However, when human and bovine adrenocortical cells were isolated from the gland and placed in primary culture, p21 increased rapidly (13). In adrenocortical cells in culture, p21 mRNA levels remained relatively constant under all conditions studied; there was a tendency for p21 to increase when the cells reached senescence but this increase was not as dramatic as that originally described for the senescence of human fibroblasts (13).

When bovine adrenocortical cells were transplanted into immunodeficient animals to form a functional tissue structure, p21 levels were initially high immediately after transplantation (16) and remained detectable in many cells for long periods. However, eventually the level of p21 in the transplant tissues declined to essentially zero, thereby resembling the normal situation in the adrenal

gland *in vivo* (M. Thomas and P.J. Hornsby, unpublished observations).

This behavior of p21 is consistent with two different hypotheses about its role in this differentiated cell type. The first is that its increase in cell culture results from damage to the cells during isolation from the intact gland (14). Continued expression in culture would indicate ongoing damage to the cells; only after long periods of time when the cells are returned to the *in vivo* environment would the damage be reversed sufficiently for the p21 level to decline. The second hypothesis is that, despite its role as a cell cycle inhibitor, p21 is elevated because the cells are dividing rather than quiescent. When cells are placed in culture and they go from their normally noncycling state *in vivo* to rapid cell division, p21 increases as a part of that process (7, 8). When the cells are transplanted into an animal, their proliferation rate decreases dramatically, although it does not become zero for a long period of time (12). Therefore, the slow decrease of p21 in bovine adrenocortical cells after transplantation may result from the exit of the cells from the cell cycle.

The present experiments in primary cultures of bovine adrenocortical cells, with additional data on primary human adrenocortical cells, provide evidence that supports the second of these two hypotheses.

METHODS

Growth of adrenocortical cells in culture

Bovine adrenocortical cells were derived by enzymatic and mechanical dispersion from the adrenal cortex of two-year-old steers, as previously described (17, 18). Human adrenocortical cells were prepared from glands from young adult kidney transplant donors by the same procedure. Primary cell suspensions were stored frozen in liquid nitrogen.

Frozen cells were thawed and replated in Dulbecco's Eagle's Medium/Ham's F-12 1:1 with 10% fetal bovine serum, 10% horse serum, 0.1 ng/ml recombinant basic FGF (R and D Systems, Minneapolis, MN) and 1% UltroSer G (Biosepra, Villeneuve-la-Garenne, France) (17, 18) at 10^5 cells per 10-cm plate.

For labeling with bromodeoxyuridine (BrdU), 30 µM BrdU was added to cultures; after one hour the cells were harvested as described below or the medium was replaced with fresh medium without BrdU when the cells were to be harvested later.

Immunocytochemistry

Cells were removed from the culture dish by proteolytic digestion (17) and were embedded in a thrombin/fibrin clot (30 µl cell pellet was mixed with 30 µl fibrin glue and 30 units topical thrombin in a total volume of 90 µl). The embedded cells were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections (6 µm) were deparaffinized and rehydrated using graded alcohol concentrations. Antigen retrieval was performed by heating at 100°C in 100 mM sodium citrate pH 6.0 for 15

min. After blocking nonspecific binding with horse serum for 30 min., sections were incubated with mouse monoclonal anti-human p21, clone EA10 (Oncogene Research Products, Cambridge, MA) at 1:20 dilution for 16 hours at 4°C. Unbound antibody was removed by washing in PBS at room temperature and then a horse anti-mouse polyclonal antibody conjugated with biotin (Vector Laboratories, Burlingame, CA) was added at 1:75 dilution for 1 hour at room temperature. The bound antibody was visualized by addition of avidin-Texas Red conjugate (Vector Laboratories) at 4 µg/ml in 50 mM sodium bicarbonate, 15 mM sodium chloride, pH 8.2, for 45 minutes. Sections were washed in the same buffer. Sections were then stained by a second antibody to visualize proliferating cells, either as incorporation of BrdU or expression of the Ki-67 proliferation-associated antigen (19). In the case of dual staining for p21 and BrdU, unbound biotin on the section was blocked by incubation in a blocking buffer as recommended by the manufacturer (Vector Laboratories). The second antibody was rat monoclonal anti-BrdU, clone BU1-75 (Harlan, Indianapolis, IN), at 1:75 dilution, followed by visualization with an avidin-fluorescein conjugate using the procedure described above. For dual staining for p21 and Ki-67, sections were incubated with a 1:10 dilution of mouse monoclonal clone MIB1 which had been directly conjugated to fluorescein (Coulter Cytometry, Miami, FL).

Before observation by fluorescence microscopy, sections were counterstained with a DNA-binding dye. Sections were incubated for 5 minutes at 4°C in 1 µg/ml 4',6-diamidino-2-phenyl indole (DAPI). After washing, sections were observed and photographed using a triple band (red/green/blue) emission filter in conjunction with single band (yellow/blue/ultraviolet) excitation filters. Numbers of nuclei labeled by the different antibodies used were counted from photographs of random fields; 1000 to 5000 cells were observed for each cell harvest timepoint.

RESULTS AND DISCUSSION

In primary culture, bovine and human adrenocortical cells show three growth phases (10): first there is a lag of about 48 hours where no growth occurs; the cells then begin DNA synthesis and proliferate rapidly; this is followed by density-dependent inhibition of growth. They can remain in a viable nondividing state for extended periods in the density-inhibited cultures. We examined levels and patterns of proliferation and p21 immunoreactivity during these growth phases. We used double-labeling techniques for the detection of p21 together with bromodeoxyuridine (BrdU) or Ki-67 as proliferation markers (Figure 1).

As expected, both BrdU incorporation and nuclear Ki-67 immunoreactivity were highest in the exponential growth phase, decreased slightly as the cells reached confluence, and declined to close to zero in the density-inhibited culture (Figure 2).
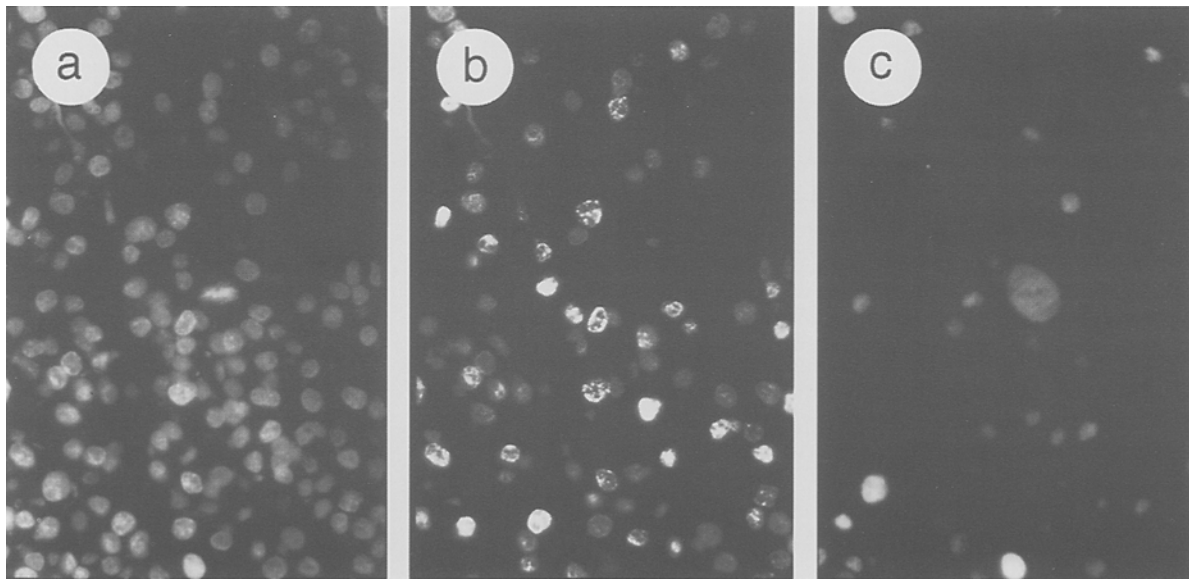
Figure 1: Detection of p21 and incorporated BrdU in exponentially proliferating bovine adrenocortical cells. Pelleted cells were fixed and sectioned for immunocytochemistry as described in Materials and Methods. **a**, the total cell population is visualized with DAPI. **b**, the same section showing incorporation of BrdU. **c**, the same section showing labeling for p21. Note that nuclei which have incorporated BrdU and those which have p21 form two separate populations. One cell that is in mitosis, as recognized by DAPI staining of chromosomes, shows cytoplasmic rather than nuclear p21. Magnification x 300.

Labeling with an anti-p21 antibody was almost entirely nuclear, except that cytoplasmic staining was noted in an occasional cell in mitosis (Figure 1). p21 was at its highest when the culture was in the exponential phase of growth and was lower when the cells became confluent (Figure 2). The percentage of p21$^+$ cells remained at ~5% in the density-inhibited state when BrdU incorporation had declined to close to zero. Double-labeling studies showed that very few cells that are in S phase in the exponentially growing culture are p21$^+$; the p21$^+$ cells are almost all non-S phase cells (Figure 2D). However, as the cells became confluent and stopped dividing, the BrdU-labeled population was much more likely to be p21$^+$. When double labeling was done for Ki-67 and p21, the patterns of labeling in the exponential, confluent and density-inhibited culture were the same, with the exception that dividing cells in the exponential phase of growth were more likely to be p21$^+$ when division was assessed by Ki-67 rather than BrdU. The greater extent of double labeling for p21 and Ki-67 than for p21 and BrdU likely reflects the fact that Ki-67 is found in the nucleus of cycling cells over more of the cell cycle than just S phase (19).

When the same three phases of growth were investigated in human adrenocortical cells we found very small numbers of p21$^+$ cells; the p21$^+$ fraction was always less than 5% and was somewhat higher in the density-inhibited state (Figure 3). The small numbers of p21$^+$ cells in exponentially growing and confluent cultures were entirely BrdU$^-$ and Ki-67$^-$. As in bovine cultures, a few cells were p21$^+$/BrdU$^+$ and p21$^+$/Ki-67$^+$ in nongrowing confluent cultures. Previously we observed that human adrenocortical cells have relatively constant p21 levels over

their life span in culture (13). While it cannot be ruled out that there is a lower level of p21 in a larger number of cells not detected by the present staining technique, it is also reasonable to conclude that the p21 levels in human adrenocortical cell cultures are partially accounted for by high levels in a relatively small percentage of the cell population. However, the levels of p21 as assessed by Northern or Western blotting seem to be about the same in human and bovine adrenocortical cell cultures (13). This reinforces the possibility that the antibody staining procedure does not have a comparable sensitivity for human and bovine cells, even though the monoclonal antibody used was raised against the human p21 protein.

In proliferating bovine adrenocortical cell cultures, are the p21$^+$ cells simply out of cycle (perhaps because of damage) or are they in fact cycling cells, but in a phase of the cell cycle other than S? We performed a time-course experiment to investigate whether cells that incorporate BrdU during S phase later become p21$^+$ (Figure 4). Cultures were labeled with BrdU at 1, 8, 16 or 24 hours before harvesting the cells. The numbers of BrdU$^+$ cells increased over the time of this experiment, presumably resulting partially from division of BrdU-labeled cells and partially from continued labeling of DNA from the intracellular pool of BrdU. Immediately after a 1-hour labeling period, only ~1% of cells were both BrdU$^+$ and p21$^+$. As the time between the 1-hour BrdU labeling period and harvesting increased, the number of double-labeled cells increased approximately 5-fold. Thus, some cells that were in S phase became p21$^+$ within the next 24 hours. However, the number of p21$^+$ cells was more or less constant over the experiment. These results are consis-
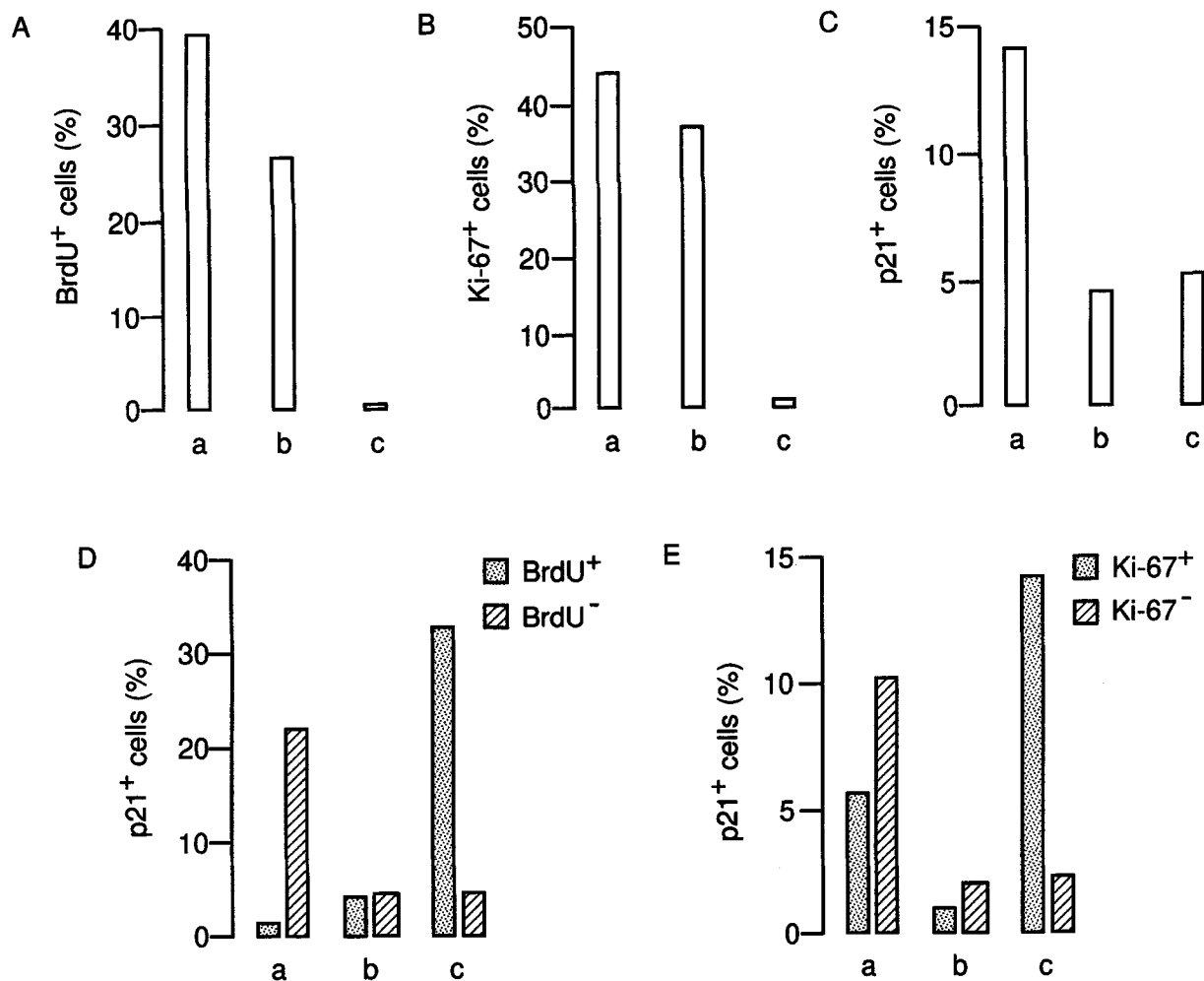
Figure 2: Comparison of incorporation of BrdU (**A**) and nuclear immunoreactivity for Ki-67 (**B**) and p21 (**C**) in proliferating bovine adrenocortical cells in culture. Cells were incubated with BrdU for 1 hour prior to harvest at three different stages of growth; **a**, cells in log phase growth at approximately 50% confluence; **b**, cells which had just reached confluence but had not yet ceased dividing; **c**, cells which had been at confluence for 7 days. **D** shows the percentages of cells with nuclear p21 in the BrdU⁺ and BrdU⁻ cell populations at the three time points at which cells were harvested. **E** shows the percentages of cells with nuclear p21 in the Ki-67⁺ and Ki-67⁻ cell populations.

tent with the hypothesis that p21⁺ cells are cycling, but not in S phase. Following one S phase, nuclear p21 increases in the G2 phase and in the following G1 phase (7, 8). During a second S phase, nuclear p21 levels would be expected to decrease again. The likelihood that many BrdU-labeled cells enter a second S phase limits the number of BrdU⁺/p21⁺ that can be detected. Double labeling would be more likely in those cells with a longer time between cycles; the cell cycle kinetics are not likely to be absolutely uniform over the entire cell population (10, 20). When a cell exits from the cycle and does not re-enter a new cycle, it is likely that p21 remains high for some period before then decreasing to very low levels. This behavior was noted in human fibroblasts at senescence, where very high p21 levels were progressively lowered after cells had been in a nondividing state for some time (21). Curiously, under confluent conditions, the small number of cells that are still cycling were much more likely to be p21⁺ than cycling cells in exponentially growing cultures. It can be speculated that such cells are traversing
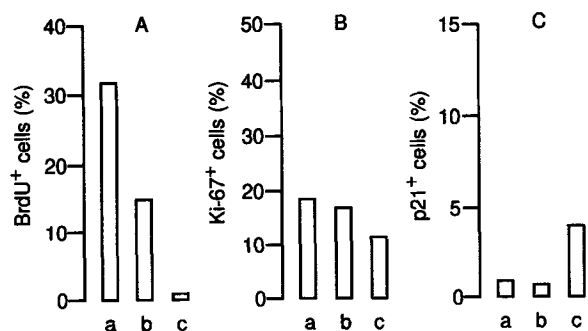

Figure 3: Percentages of cells incorporating BrdU (**A**) and immunoreactive for Ki-67 (**B**) and p21 (**C**) at three phases of growth in human adrenocortical cells in culture. Cells were harvested at the same time points as for the bovine cells in Figure 2 and were processed for histochemistry in the same way.
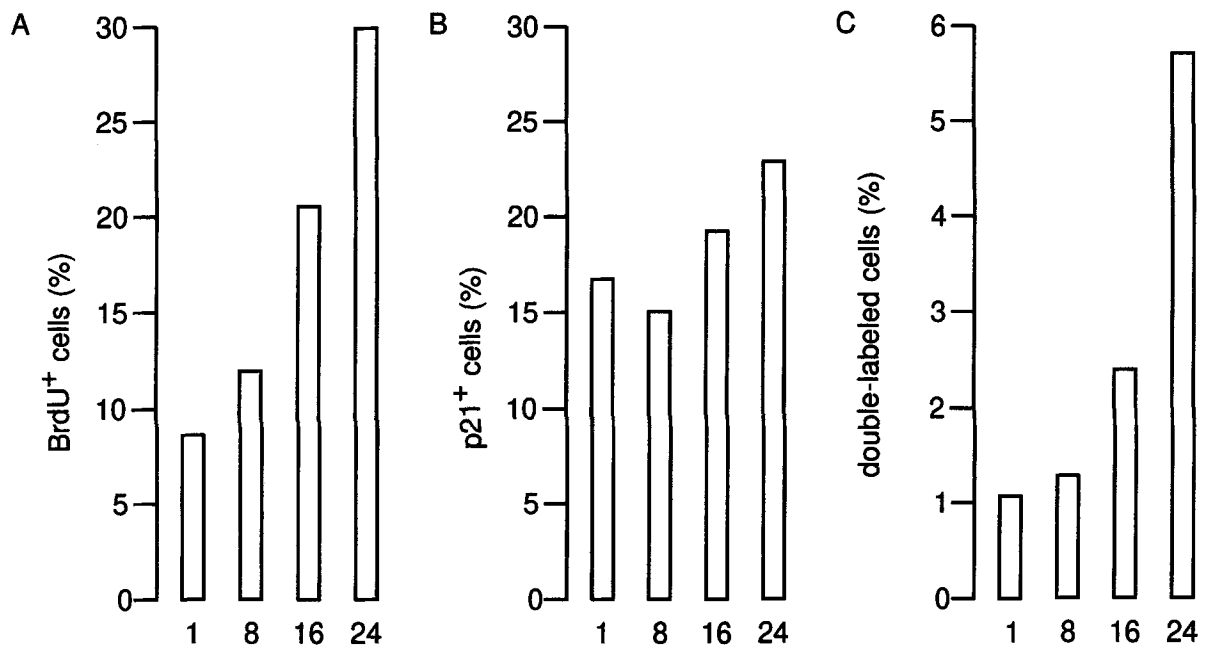
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Figure 4: Time course experiment showing nuclear p21 immunoreactivity in bovine adrenocortical cells labeled with BrdU. BrdU was added to the cells to confluent, but still dividing cultures and was removed after a 1-hour incubation. The cells were harvested at the indicated times (1, 8, 16, or 24 hours) following BrdU addition. The figure shows (**A**) percentages of cells labeled with BrdU; (**B**) the percentages of cells in the same section with nuclear p21, and (**C**) the percentage of cells in the sections that are positive for both BrdU and p21.

a final cell cycle before exiting from the cell cycle because of the inhibition of cell division that occurs under crowded conditions in culture, but it is not clear how they are able to divide with high p21. Although p21 was originally described as a protein expressed at high levels in senescent fibroblasts (1, 4), in adrenocortical cells p21 was detected throughout growth phases in culture and did not show the very large increase at senescence originally observed in fibroblasts (13). It may be that senescence does cause increased transcription of p21 in this cell type, but this increase is not readily detectable when levels of p21 are already being raised by other mechanisms.

These data support a role for p21 in regulating the cell cycle in dividing adrenocortical cells, but other cell cycle inhibitors may be responsible for maintaining cells in a nondividing quiescent state both in culture and *in vivo* (21 - 24). These experiments do not suggest that p21 was associated with damage of cells. On the other hand, there clearly is a role for p21 as a response to DNA damage in adrenocortical cells, both in culture and *in vivo*. In the intact adrenal gland, DNA damage rapidly increases p21 throughout the adrenal cortex, but this is not associated either positively or negatively with cell division (14, 15).

We have recently been able to compare the behavior of p21 in bovine adrenocortical cells in culture and after transplantation into scid mice (11, 12). In the tissue that forms from cells transplanted *in vivo*, cells in the region of the tissue where cell division is occurring have nuclear p21 immunoreactivity whereas the region that is damaged and dying is p21⁻ as well as Ki-67⁻ (16). In long-term

transplants of bovine adrenocortical cells proliferation declines to almost zero; in these tissues p21+ cells are very rare (M. Thomas and P.J.H., unpublished observations). These long-term transplant tissues therefore resemble the normal bovine adrenal cortex *in vivo*, which has low proliferation and almost no expression of p21 (14). An increase in p21 was also noted immediately after transplantation, which does not appear to be associated with cell division, but may well be associated with DNA damage (16). However this is a transient response, and after a few days p21+ cells are exclusively found in the healthy dividing areas of the transplant tissue.

Thus, despite a clear role for p21 as a response to damage in adrenocortical cells, the major role of p21 in primary cell cultures as well as in transplants appears to be to regulate the cell cycle in the fraction of the population that is undergoing cell division. The full role of p21 in cell biology still remains to be determined, and the possible importance of p21 as a regulator of the cell cycle has to be balanced against the rather mild phenotype of the p21 knockout mouse (22). The adrenocortical cell system, which uniquely combines *in vitro* and *in vivo* experiments on the same cell type, may play a role in the future elucidation of these questions. Moreover, p21 clearly has multiple roles and high levels of p21 cannot be used as a molecular indicator of senescence in cells. The ability to study adrenocortical cells in both cell culture and in a cell transplantation model may reveal other molecular features which characterize the senescent state of cells both *in vitro* and *in vivo*.

REFERENCES

1. Smith, JR: Inhibitors of DNA synthesis derived from senescent human diploid fibroblasts. Exp. Gerontol., 27: 409-12, 1992.

2. El-Deiry, WS, Tokino, T, Velculescu, VE, Levy, DB, Parsons, R, Trent, JM, Lin, D, Mercer, WE, Kinzler, KW, and Vogelstein, B: WAF1, a potential mediator of p53 tumor suppression. Cell, 75: 817-825, 1993.

3. Harper, JW, Elledge, SJ, Keyomarsi, K, Dynlacht, B, Tsai, L-H, Zhang, P, Dobrowolski, S, Bai, C, Connell-Crowley, L, Swindell, E, Fox, MP, and Wei, N: Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell, 6: 387-400, 1995.

4. Noda, A, Ning, Y, Venable, SF, Pereira-Smith, OM, and Smith, JR: Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res., 211: 90-98, 1994.

5. Levine, AJ: p53, the cellular gatekeeper for growth and division. Cell, 88: 323-31, 1997.

6. Paulovich, AG, Toczyski, DP, and Hartwell, LH: When checkpoints fail. Cell, 88: 315-21, 1997.

7. Dulic, V, Stein, GH, Far, DF, and Reed, SI: Nuclear accumulation of p21^{Cip1} at the onset of mitosis: A role at the G2/M-phase transition. Mol. Cell. Biol., 18: 546-557, 1998.

8. Niculescu, AB, Chen, XB, Smeets, M, Hengst, L, Prives, C, and Reed, SI: Effects of p21$^{Cip1/Waf1}$ at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol. Cell. Biol., 18: 629-643, 1998.

9. El-Deiry, WS, Tokino, T, Waldman, T, Oliner, JD, Velculescu, VE, Burrell, M, Hill, DE, Healy, E, Rees, JL, Hamilton, SR, Kinzler, KW, and Vogelstein, B: Topological control of p21$^{WAF1/CIP1}$ expression in normal and neoplastic tissues. Cancer Res., 55: 2910-2919, 1995.

10. Hornsby, PJ, Cheng, CY, Lala, DS, Maghsoudlou, SS, Raju, SG, and Yang, L: Changes in gene expression during senescence of adrenocortical cells in culture. J. Steroid Biochem. Mol. Biol., 43: 385-395, 1992.

11. Thomas, M, Northrup, SR, and Hornsby, PJ: Adrenocortical tissue formed by transplantation of normal clones of bovine adrenocortical cells in scid mice replaces the essential functions of the animals' adrenal glands. Nature Med., 3: 978-983, 1997.

12. Hornsby, PJ, Thomas, M, Northrup, SR, Popnikolov, NP, Wang, X, Tunstead, JR, and Zheng, J: Cell transplantation: A tool to study adrenocortical cell biology, physiology, and senescence. Endocr. Res., 24: 909-918, 1998.

13. Yang, L, Didenko, VV, Noda, A, Bilyeu, TA, Darlington, GD, Smith, JR, and Hornsby, PJ: Increased expression of p21^{Sdi1} in adrenocortical cells when they are placed in culture. Exp. Cell Res., 221: 126-131, 1995.

14. Didenko, VV, Wang, X, Yang, L, and Hornsby, PJ: Expression of p21$^{WAF1/CIP1/SDI1}$ and p53 in apoptotic cells in the adrenal cortex and induction by ischemia/reperfusion injury. J. Clin. Invest., 97: 1723-1731, 1996.

15. Didenko, VV, Wang, X, Yang, L, and Hornsby, PJ: p21 and apoptosis in experimental damage of the rat adrenal cortex and trauma-associated damage of the human adrenal cortex. J. Pathol. (in press), 1999.

16. Tunstead, JR, and Hornsby, PJ: Early events in the formation of a tissue structure from dispersed bovine adrenocortical cells following transplantation into scid mice. (submitted), 1999.

17. Hornsby, PJ, and McAllister, JM: Culturing steroidogenic cells, in Methods in Enzymology, Vol. 206, edited by Waterman, MR, and Johnson, EF, San Diego, Academic, 1991, pp. 371-380.

18. Hornsby, PJ: Culturing steroidogenic cells, in Cell and Tissue Culture: Laboratory Procedures, 17B, edited by Griffiths, JB, Doyle, A, and Newell, DG, Chichester, England, Wiley, 1994, pp. 7.1-7.9.

19. Yu, CC, Woods, AL, and Levison, DA: The assessment of cellular proliferation by immunohistochemistry: A review of currently available methods and their applications. Histochem. J., 24: 121-31, 1992.

20. Hornsby, PJ, and Yang, L: A model for phenotypic switching in adrenocortical cells senescing in culture. Mech. Ageing Dev., 58: 1-12, 1991.

21. Alcorta, DA, Xiong, Y, Phelps, D, Hannon, G, Beach, D, and Barrett, JC: Involvement of the cyclin-dependent kinase inhibitor p16^{INK4a} in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci. USA, 93: 13742-13747, 1996.

22. Deng, C, Zhang, P, Harper, JW, Elledge, SJ, and Leder, P: Mice lacking p21$^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. Cell, 82: 675-684, 1995.

23. Nakayama, K, Ishida, N, Shirane, M, Inomata, A, Inoue, T, Shishido, N, Horii, I, Loh, DY, and Nakayama, K: Mice lacking p27^{Kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell, 85: 707-20, 1996.

24. Zhang, PM, Liegeois, NJ, Wong, C, Finegold, M, Hou, H, Thompson, JC, Silverman, A, Harper, JW, DePinho, RA, and Elledge, SJ: Altered cell differentiation and proliferation in mice lacking p57^{Kip2} indicates a role in Beckwith-Wiedemann-syndrome. Nature, 387: 151-158, 1997.